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Parathyroid hormone prevents $1,25(OH)_2D_3$ induced down-regulation of the vitamin D receptor in growth plate chondrocytes *in vitro*

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Parathyroid hormone prevents $1,25(OH)_2D_3$ induced down-regulation of the vitamin D receptor in growth plate chondrocytes *in vitro*. $1,25(OH)_2D_3$ has an antiproliferative effect on growth plate chondrocytes when given in high doses, whereas low doses stimulate chondrocyte proliferation. In the present *in vitro* study we investigated the effects of parathyroid hormone (PTH) when given concomitantly with $1,25(OH)_2D_3$ on cell proliferation and vitamin D receptor (VDR) regulation. Freshly isolated rat tibial chondrocytes were grown in monolayer cultures or in agarose stabilized suspension cultures (10% charcoal-treated FCS). VDR expression was determined by RT-PCR generating a 297 bp fragment and by binding assays (Scatchard analysis) with [^{3}H]-$1,25(OH)_2D_3$. Cell proliferation was measured by [^{3}H]-thymidine incorporation, growth curves in monolayer cultures and by colony formation in agarose-stabilized suspension cultures. Optimal concentration of $1,25(OH)_2D_3$ (10^{-12} M) and of PTH fragments [bPTH(1-34) or hPTH(28–48), 10^{-10} M] showed additive effects on DNA synthesis of and colony formation by growth plate chondrocytes. This may be explained in part by an up-regulation of VDR by PTH: PTH increased both mRNA expression of VDR and binding capacity. $1,25(OH)_2D_3$ (10^{-12} M) induced an up-regulation of the VDR within 24 hours followed by a down-regulation after incubation for more than 24 hours. PTH fragments added concomitantly prevented the down-regulation seen with $1,25(OH)_2D_3$. These findings provide evidence that PTH is a growth promoting hormone that also modulates the effects of $1,25(OH)_2D_3$ by regulating the VDR status of $1,25(OH)_2D_3$ target cells.

In children with chronic renal failure, disturbed vitamin D metabolism and secondary hyperparathyroidism contribute to growth failure [1–3]. $1,25(OH)_2D_3$ and parathyroid hormone (PTH) interact to form an endocrine feedback mechanism: $1,25(OH)_2D_3$ suppresses mRNA for pre-pro-PTH [4, 5] and inhibits proliferation of parathyroid cells [6]. Conversely, PTH is a potent stimulator of renal 1α-hydroxylase [7].

PTH and $1,25(OH)_2D_3$ control growth by their direct actions on bone and cartilage cells [8–13]. In earlier studies, we demonstrated that $1,25(OH)_2D_3$ had a biphasic effect on proliferation of growth cartilage cells; proliferation is stimulated by $1,25(OH)_2D_3$ at a concentration of 10^{-12} M, but not at high concentrations (10^{-8} M) [9]. Long-term exposure of growth cartilage cells to $1,25(OH)_2D_3$ results in a homologous down-regulation of the VDR [14]. This effect may potentially reduce the biological activity of $1,25(OH)_2D_3$ on its target cells.

In the present study we investigated whether PTH is able (*i*) to overcome the $1,25(OH)_2D_3$-induced down-regulation of the VDR in growth plate chondrocytes, and (*ii*) to modulate the effect of $1,25(OH)_2D_3$ on DNA synthesis and colony formation by chondrocytes.

Key words: Vitamin D, $1,25(OH)_2D_3$, PTH, cartilage.

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METHODS

Materials

$1,25(OH)_2$-[26,27-methyl-^{3}H]cholecalciferol (158 Ci/mmol) was obtained from Amersham Buchler (Braunschweig, Germany); unlabeled $1\alpha,25(OH)_2D_3$, $25(OH)D_3$ were gifts from Dr. Calcanis and Dr. Uskokovic (Hoffmann-La Roche, Germany and USA); hydroxyapatite, dithiothreitol, Triton X-100, were from Sigma Chemical Co. (Munich, Germany); F-12 medium, DMEM (Dulbecco's modified minimal essential medium), PBS (phosphate buffered saline), gentamicin sulphate, clostridium collagenase (EC 3.4.24.3), DNAse I (EC 3.1.21.1), and trypan blue were from Boehringer (Mannheim, Germany). The bovine PTH-fragment (1-34) [bPTH(1-34)] were from Bissendorf-Biochemicals (Hannover, Germany), the human PTH-fragment (28-48) [hPTH(28-48)] was synthesized at the Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany [10]. Standard low (SL)- and Low gel temperature (LGT)-agarose was purchased from BioRad (Richmond, VA, USA). FCS was from Seromed (Berlin, Germany); Primers and Superscript II reverse transcriptase were purchased from Gibco BRL (Eggenheim, Germany). $1,25(OH)_2D_3$ in FCS determined by radioimmunoassay [15] was 1.1×10^{-10} M; Charcoal treatment reduced $1\alpha,25(OH)_2D_3$ concentrations below the detection limit of the $1\alpha,25(OH)_2D_3$ assay (2.4×10^{-11} M). PTH related peptide (PTHrP) in charcoal-stripped FCS was 9,8 pg/ml (radioimmunoassay, Fa. Immundiagnostik, Bensheim, Germany).

Cell cultures

Isolation of chondrocytes. Epiphyseal chondrocytes from 80 g Sprague-Dawley rats (Charles River, Kieslegg, Germany) were

isolated and cultured with a modified method of Benya and Shaffer [16] as described [9]. In brief, the epiphyseal growth plates (tibia) were cleaned of perichondrium and dissected microscopically by a transverse cut through the calcification zone and by separation from the epiphysis with a scalpel, carefully avoiding any loss of cartilage close to the bony epiphysis.

Pooled growth plates from 5 (monolayer or agarose stabilized suspension cultures) or 10 (VDR-assays) animals were digested for three hours at 37°C by clostridial collagenase (0.12% wt/vol, Fa. Boehringer) and 0.02% wt/vol bacterial DNAse (Fa. Boehringer) in F-12/DMEM 1/1 medium. After isolation cells were washed three times in serum free medium. Viability, determined by the trypan blue exclusion technique, always exceeded 90%. Dissociated cells were counted using a Neubauer chamber (W. Schreck, Hofheim, Germany).

Monolayer cultures. Cells were cultured in 60 mm plastic wells (Falcon Labware, Oxnard, CA, USA) for proliferation assays and in 100 mm dishes for VDR-determination using F-12/DMEM 1/1 medium, supplemented with 50 μg/ml L-ascorbic acid, 10 mmol/liter HEPES, 100 μg/ml gentamicin and 10% fetal calf serum (FCS) or 10% charcoal-stripped FCS (Ch-FCS) at 37°C, gassed with 95% air/5% CO_2. The nominal calcium concentration, measured with an ion-selective electrode of a Fresenius Ionometer EF (Fresenius, Oberursel, Germany) was 1.2 mmol/liter. PTH-fragments dissolved in phosphate buffered saline (PBS) and/or vitamin D metabolites dissolved in ethanol or vehicle control (ethanol, 0.05% final concentration) were added with fresh medium every other day unless otherwise stated. Cells reached a saturation density of 1.2×10^5 cells/cm^2.

Agarose stabilized suspension cultures

Cells were cultured in agarose according to Benya and Schaffer [16] as described [9]. Sixty mm Petri dishes were coated with a bottom film of 1% standard low (SL) agarose in water. The agarose was autoclaved (110°C, 45 min) before coating and was allowed to solidify at room temperature. Low gel temperature (LGT) agarose was autoclaved as above, and mixed with an equal volume of double concentrated F-12/DMEM 1/1 medium to give a final concentration of agarose of 1%. The cells were diluted with F-12/DMEM 1/1 medium, containing 10% Ch-FCS at 37°C to a final concentration of 160,000 cells/ml. This cell suspension was then mixed with 1% LGT agarose (37°C) to give a final cell concentration of 80,000 cells/ml in 0.5% LGT. Precoated dishes were heated to 37°C before 2 ml of the LGT-cell suspension were added. Dishes were kept at 37°C for 10 minutes before gelation was obtained at 4°C (10 min). Subsequently, 3 ml F-12/DMEM 1/1 medium supplemented with 10% Ch-FCS and various concentrations of PTH-fragments and/or vitamin D metabolites (or solvent control) as indicated, were added on top of the gelated agarose. Medium was changed every other day. The cultures were screened for adherent cell clusters of more than five cells. No such clusters were seen at the start of culture in any experiment presented in this study.

Immunocytology for PCNA. Freshly isolated chondrocytes were plated on glass slides in 35 mm dishes in F-12/DMEM 1/1 medium, nominal calcium concentration 1.2 mmol/liter supplemented with 10% FCS. Medium was changed every other day, until cells reached subconfluency (day 4). For synchronization of cell cycles, cells were kept in serum-free F-12/DMEM 1/1 medium for 24 hours as described [9]. Thereafter the medium was changed to F-12/DMEM 1/1, 1.2 mmol/liter calcium supplemented with 10% Ch-FCS. bPTH(1-34) was added for the first six hours of the culture period, followed by thoroughly washes with medium and a postincubation in the absence of bPTH(1-34). Under these conditions, best results with respect to PTH-driven [^{3}H]-thymidine-incorporation were obtained [13]. The expression of PCNA (proliferating cell nuclear antigen) was examined in cells fixated in formalin (3.7%, 7 min). Afterwards the specimen was bathed in Carnoy sp. (ethanol/acetate 2/1 vol/vol) for five minutes, followed by three washes with PBS. The rabbit anti mouse monoclonal antibody E-413 (Fa. DAKO, Denmark) was diluted 1:400. Visualization was obtained by the streptavidin-biotin method as described [17].

Assays of chondrocyte growth and proliferation

Radiothymidine incorporation. Incorporation of [^{3}H]-thymidine into DNA was determined as radioactivity in TCA-precipitable material as described [9]. Prior to the start of experiments cells were synchronized by maintaining them under serum free conditions for 24 hours. Medium was changed (10% Ch-FCS) and hormones or solvent added as indicated for 8 to 48 hours. Two μCi of [^{3}H]-thymidine were added for the last four hours. Cells were counted in parallel cultures.

Colony formation in agarose stabilized suspension cultures. Suspension cultures were terminated by fixation in buffered formaldehyde (4%). Colonies were counted in 100 squares (2 mm grid) for each dish. A cell colony was defined as a cluster of more than five cells. For easier identification of colonies, the formed matrix was stained by alcian blue under acidic condition as described [9, 18]. These staining technique visualizes glycoaminoglycans. Data are given as colony count and as cloning efficiency, that is, colonies formed per 1000 seeded cells.

RT-PCR

RNA preparation. Total cellular RNA was collected to examine the expression of VDR receptor. Total RNA from cultured cells was isolated by the RNA-Clean® method (Angewandte Gentechnologie Systeme, Heidelberg, Germany) following the instructions of the manufacturers.

RT-PCR amplification of VDR. RT and PCR primers were deduced from rat VDR sequence [19]. Two micrograms of total RNA were reverse transcribed into cDNA with Superscript reverse transcriptase and specific primers for VDR (R1, 5′-CCGAACACCTCCAGCACAAG-3′). After cDNA synthesis, excess primers were removed and PCR amplification was performed using the cDNA template with the specific primers pairs (F1, 5′-GCCCACCACAAGACCTAT-3′; and R2, 5′-CCTTTTG-GAT-GCTGTAACTG-3′). The amplification profile in a Perkin Palmer Gene Amp PCR System 9600 consisted of denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds, and extension at 72°C for 30 seconds for all primers after a five-minute denaturation step at 94°C. The 32 cycle amplified products (297 bp for VDR) were detected by electrophoresis in a 2% agarose gel and visualized by ethidium bromide staining and ultraviolet transillumination. As control, β-actin mRNA was also reverse transcribed using specific primer (R1, 5′-CTAGAAGCATTT-GCGGTCGGAC-3′) and the resulting cDNA amplified with the primers β-actin R1 and β-actin F1 (5′-CATCACCATTG-GCAATGAGCG-3′) following the same protocol resulting in a PCR product of 405 bp. Control reactions performed by omitting

Table 1. Effect of bPTH(1-34), $1,25(OH)_2D_3$ and their combination on radiothymidine incorporation in monolayer cultures

$1,25(OH)_2D_3$ added	bPTH(1-34) added	Radiothymidine incorporation *% of solvent control*
Solvent	Solvent	100 ± 13
	10^{-12} M	105 ± 12
	10^{-10} M	162 ± 18[a,b]
	10^{-8} M	143 ± 9[a]
10^{-12} M		200 ± 5[a,c]
10^{-10} M		154 ± 6[a]
10^{-8} M		93 ± 5
10^{-12} M	10^{-10} M	257 ± 15[a,d]
10^{-10} M	10^{-10} M	226 ± 15[a,e]
10^{-8} M	10^{-10} M	120 ± 13

Cell cycles of subconfluent chondrocyte cultures were synchronized by incubation in serum-free medium for 24 hours. After changing the medium to F12/DMEM 1/1 with 10% Ch-FCS, bPTH(1-34) was added at indicated concentrations for 6 hours. After three washes, postincubation was performed in the absence of bPTH(1-34) for 42 hours. Cells stimulated with $1,25(OH)_2D_3$ (10^{-12} M to 10^{-8} M) with or without PTH were incubated with the steroid hormone for the total culture period, that is, $1,25(OH)_2D_3$ was again added after the washes. Radiothymidine was added for the last 4 hours of the 48 hour incubation period. Statistics are by ANOVA, $N = 8$ per group.

[a] $P < 0.01$ vs. solvent control
[b] $P < 0.01$ vs. bPTH 10^{-8} M
[c] $P < 0.01$ vs. $1,25(OH)_2D_3$ 10^{-10} M
[d] $P < 0.01$ vs. $1,25(OH)_2D_3$ 10^{-12} M and bPTH(1-34) 10^{-10} M
[e] $P < 0.01$ vs. $1,25(OH)_2D_3$ 10^{-10} M and bPTH(1-34) 10^{-10} M

reverse transcriptase or template RNA showed no reaction product. The optic densities of the VDR product was normalized for the density of β-actin by a commercially available computer software program (Bio-1D V.96.; Vilber, Lourmat, France).

Assay of $1,25(OH)_2D_3$ receptor (VDR) activity

KTED extracts. Cell extracts were prepared as described elsewhere [20]. In brief, cell suspensions (1×10^7 cells/ml) were homogenized in 0.4 M KTED buffer (0.4 M KCl, 10 mM Tris HCl, 1,5 mM EDTA, 2 mM dithiothreitol, 10 mM sodium molybdate, pH 7,4). A purified fraction was prepared by centrifugation at 205,000 g for 30 minutes using a Ti-50 rotor (Beckman Instruments, Fullerton, CA, USA). The supernatant was used for binding studies.

Scatchard analysis. Saturation analysis according to Scatchard [21] was carried out as described [9]. One hundred microliter aliquots (protein concentration 0.5 to 1.2 mg/ml) were incubated for 16 hours at 4°C with increasing concentrations (0.1 to 7.0 nM) of [^{3}H]-$1,25(OH)_2D_3$ in the absence or presence of 100-fold molar excess of $1,25(OH)_2D_3$. Bound [^{3}H]-$1,25(OH)_2D_3$ was determined using the hydroxyapatite assay [22].

Statistics

Data are given as mean ± SD. Statistical analysis was carried out using ANOVA or the Mann-Whitney-*U*-test as appropriate with a commercial available PC-software (Statgraph Plus, Statgraphics Cooperation, USA and Sigma Stat, Jansen, Germany). Statistical significance was considered for $P < 0.05$.

Table 2. Effect of bPTH(1-34), $1,25(OH)_2D_3$ and their combination on colony formation and cloning efficiency

Hormone added	Colony formation *% of solvent control*	Cloning efficiency *colonies/1000 seeded cells*
A		
Solvent control	100 ± 11.7	1.14 ± 0.24
$1,25(OH)_2D_3$ 10^{-12} M	164 ± 21.6[a]	1.87 ± 0.40[a]
$1,25(OH)_2D_3$ 10^{-10} M	87.9 ± 20.3	0.99 ± 0.20
$1,25(OH)_2D_3$ 10^{-8} M	82.0 ± 22.6	0.94 ± 0.20
$1,25(OH)_2D_3$ 10^{-6} M	73.8 ± 19.4	0.84 ± 0.16
B		
Solvent control	100 ± 14.5	0.91 ± 0.06
bPTH(1-34) 10^{-10} M	187 ± 14.3[a]	1.71 ± 0.03[b]
bPTH(1-34) 10^{-8} M	162 ± 6.2[a]	1.47 ± 0.06[b]
bPTH(1-34) 10^{-6} M	128 ± 9.4	1.16 ± 0.08
C		
Solvent control	100 ± 15.6	1.27 ± 0.21
$1,25(OH)_2D_3$ 10^{-12} M	152 ± 14.2[a]	1.75 ± 0.25[a]
bPTH(1-34) 10^{-10} M	149 ± 10.8[a]	1.85 ± 0.20[a]
$1,25(OH)_2D_3$ + bPTH(1-34)	194 ± 10.9[a,c]	2.75 ± 0.30[a,c]

[a] $P < 0.03$ vs. solvent control
[b] $P < 0.01$ vs. solvent control
[c] $P < 0.01$ vs. $1\alpha,25(OH)_2D_3$ or bPTH(1-34), respectively

RESULTS

Effect of bPTH(1-34), $1,25(OH)_2D_3$ or their combination on radiothymidine incorporation in monolayer and cloning efficiency in agarose-stabilized suspension cultures

Monolayer cultures (Table 1). Bovine PTH(1-34) dose-dependently stimulated radiothymidine incorporation in monolayer cultures of growth plate chondrocytes with an optimal effect at 10^{-10} M. Higher concentrations were less stimulatory. $1,25(OH)_2D_3$ modulated radiothymidine incorporation in a biphasic manner: low concentrations (10^{-12} M) increased DNA synthesis, whereas concentrations of 10^{-8} M or higher showed an inhibitory effect. The biphasic effect was noted when $1,25(OH)_2D_3$ was added to medium supplemented with 10% charcoal-stripped FCS or to serum-free medium (data not shown), demonstrating that a very low concentration (10^{-12} M) of $1,25(OH)_2D_3$ is effective for stimulation of DNA synthesis. Coincubation of bPTH(1-34) (10^{-10} M) and $1,25(OH)_2D_3$ (10^{-12} M to 10^{-8} M) yielded additive effects on radiothymidine incorporation for the stimulatory concentrations of $1,25(OH)_2D_3$ (10^{-12} M to 10^{-10} M); the high dose of $1,25(OH)_2D_3$ (10^{-8} M) inhibited not only basal radiothymidine incorporation, but also the effect of the PTH fragment (Table 1).

Agarose stabilized suspension cultures (Table 2). In parallel to results obtained in the monolayer culture system, $1,25(OH)_2D_3$ showed a biphasic effect on colony formation in agarose stabilized suspension cultures: a stimulation of colony formation (and cloning efficiency) was seen with 10^{-12} M $1,25(OH)_2D_3$, whereas higher concentrations up to 10^{-6} M tended to inhibit colony formation; however, this inhibitory effect narrowly missed statistical significance (Table 2). Bovine PTH(1-34) showed the highest cloning efficiency for 10^{-10} M with a monotonous decrease for higher concentrations (Table 2). In the following experiments, the optimal stimulatory concentrations of bPTH(1-34) and $1,25(OH)_2D_3$, that is, 10^{-10} M and 10^{-12} M, respectively, were used. When chondrocytes were coincubated with bPTH(1-34)

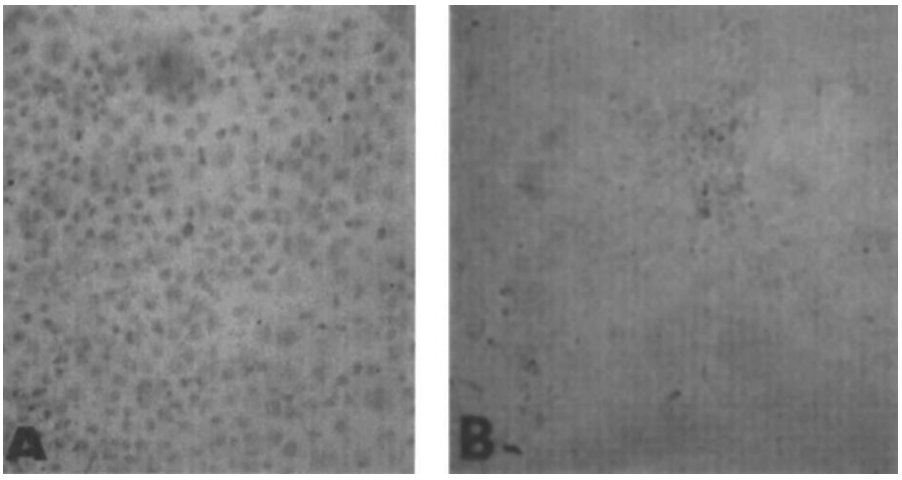

Fig. 1. Effect of bPTH(1-34) on expression of PCNA in chondrocytes. Subconfluent chondrocyte cultures were kept in serum-free F-12/DMEM 1/1 medium for 24 hours for synchronization of cell cycles. Thereafter medium was changed to F-12/DMEM 1/1, 1.2 mmol/liter calcium supplemented with 10% Ch-FCS. (*A*) Bovine PTH(1-34) was added for the first six hours of the culture period, followed by thoroughly washes with medium (10% Ch-FCS) and a post-incubation in the absence of bPTH(1-34) up to 48 hours. (*B*) Solvent control cells (10% Ch-FCS); cultures received the same washings parallel to group A. PCNA was stained immunocytochemically as described in **Methods** (original magnification ×360).

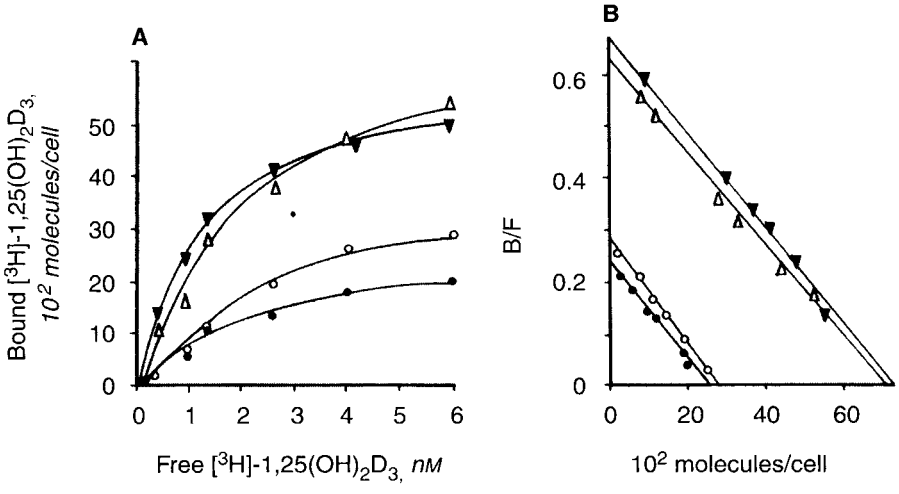

Fig. 2. Stimulation of specific binding of $[^3H]$-1,25$(OH)_2D_3$ by incubation of chondrocytes with 1,25$(OH)_2D_3$ (10^{-12} M) and/or bPTH(1-34) (10^{-10} M) after 48 hours of incubation. (*A*) Subconfluent chondrocytes, synchronized by preculture in serum-free medium for 24 hours [and by implication in the absence of 1,25$(OH)_2D_3$] were cultured in F-12/DMEM 1/1 medium, 1.2 mmol nominal calcium concentration with 10% Ch-FCS. 1,25$(OH)_2D_3$ (10^{-12} M final concentration) or bPTH(1-34) (10^{-10} M) were added for the entire 48-hour culture period. Specific binding of $[^3H]$-1,25$(OH)_2D_3$ was determined in KTED whole cell extracts as described in the **Methods** section. (*B*) The Scatchard plot shows increased specific binding capacity (intercept on the abscissa) with exposure to the hormones without any change in affinity (slope) N_{max} solvent control 2751 bound molecules/cell, 1,25$(OH)_2D_3$ 2889; bPTH(1-34) 7577; 1,25$(OH)_2D_3$ + bPTH(1-34) 7839; K_d 2.7 × 10^{-10} M, 3.4 × 10^{-10} M, 2.4 × 10^{-10} M and 4.5 × 10^{-10} M, respectively. Symbols are: (△) bPTH(1-34); (▼) bPTH(1-34) + 1, 25$(OH)_2D_3$; (○) continuous 1,25$(OH)_2D_3$; (●) control.

(10^{-10} M) and 1,25$(OH)_2D_3$ (10^{-12} M) an additive effect on cloning efficiency was demonstrable (Table 2).

Modulation of 1,25$(OH)_2D_3$-receptor expression by bPTH(1-34), 1,25$(OH)_2D_3$ or their combination

For the determination of VDR expression the monolayer culture technique was used. In these cultures, bPTH(1-34) stimulated proliferation of the majority of cultured chondrocytes and not only a subgroup, as shown by expression of PCNA (Fig. 1).

The effect of bPTH(1-34) 10^{-10} M on specific binding of $[^3H]$-1,25$(OH)_2D_3$ was measured in purified KTED whole cell extracts of subconfluent chondrocytes. Bovine PTH(1-34) increased maximal specific binding (N_{max}) three- to fourfold versus solvent control after 48 hours (Fig. 2). This increase in VDR was time dependent, reaching a plateau level after 24 hours (Fig. 3). The increased N_{max} persisted for at least 48 hours (Fig. 3). The apparent dissociation constant (K_d) was similar in all groups [solvent control, 4.2 × 10^{-10} M; bPTH(1-34) 8 hr, 2.2; bPTH(1-34) 12 hr, 2.2; bPTH(1-34) 24 hr, 2.1; bPTH(1-34) 48 hr, 3.3]. The up-regulation of the VDR on binding levels was dependent on transcriptional and translational processes, because the bPTH(1-34) driven increase in specific binding of $[^3H]$-1,25$(OH)_2D_3$ was obliterated by actinomycin D (2 μg/ml) and cycloheximide (5 μg/ml), respectively [solvent control 2678 bound molecules/cell; bPTH(1-34) 10^{-10} M 7839; actinomycin D (2 μg/ml) 2915; bPTH (1-34) + actinomycin D 3971; cycloheximide (2 μg/ml) 3015; bPTH(1-34) + cycloheximide 3080], without change in affinity (data not given).

1,25$(OH)_2D_3$ (10^{-12} M) showed a time-dependent biphasic effect on VDR expression in cultured chondrocytes. Within the first 24 hours it increased specific binding of $[^3H]$-1,25$(OH)_2D_3$ (Figs. 2 and 3). The maximal binding capacity was reached after 24 hours; with prolonged incubation with 1,25$(OH)_2D_3$ (10^{-12} M) for more than 24 hours, the binding of $[^3H]$-1,25$(OH)_2D_3$ decreased to basal levels. Chondrocytes were vital according to trypan blue exclusion tests at all time points studied. The apparent loss of

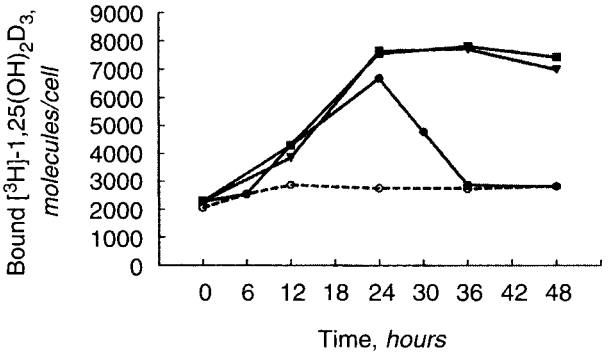

Fig. 3. Time course of $1,25(OH)_2D_3$ receptor expression by cultured chondrocytes: Effects of $1,25(OH)_2D_3$, bPTH(1-34) and their combination. Subconfluent chondrocytes, synchronized by preculture in serum-free medium for 24 hours were cultured in F-12/DMEM 1/1 medium, 1.2 mmol nominal calcium concentration with 10% Ch-FCS. $1,25(OH)_2D_3$ (10^{-12} M final concentration) or bPTH(1-34) (10^{-10} M final concentration) or their combination were added for the indicated culture periods. Specific binding of [^{3}H]-$1,25(OH)_2D_3$ was determined in KTED whole cell extracts at indicated intervals. The graph shows specific binding of [^{3}H]-$1,25(OH)_2D_3$ as a function of duration of incubation. Dissociation constants, K_d, were similar in all groups. Symbols are: (○) control; (●) $1,25(OH)_2D_3$; (▼) PTH; (■) PTH + $1,25(OH)_2D_3$.

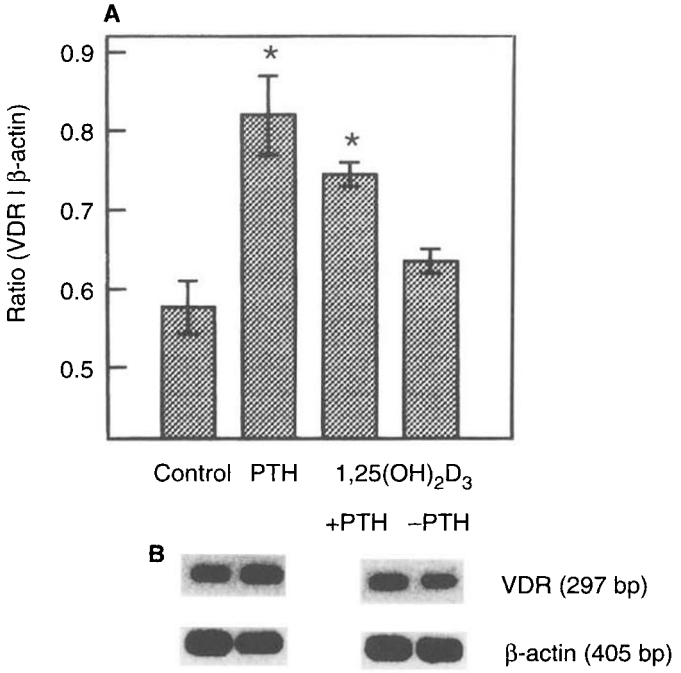

Fig. 4. Regulation of VDR mRNA by bPTH(1-34), $1,25(OH)_2D_3$ and their combination. Subconfluent chondrocyte monolayer cultures were synchronised in serum free medium for 24 hours. Medium was then changed to F12/DMEM 1/1 containing 10% Ch-FCS. Cells were incubated with bPTH(1-34) 10^{-10} M, $1,25(OH)_2D_3$ 10^{-12} M or both hormones for 24 hours. Thereafter, total RNA was prepared and mRNA levels encoding the VDR were quantitated by RT-PCR assay as described in the **Methods** section. (*A*) The graph illustrates up-regulation of the VDR mRNA by bPTH(1-34) (10^{-10} M, PTH) and by concomitant incubation with $1,25(OH)_2D_3$ (10^{-12} M) and bPTH(1-34) (10^{-10} M). $1,25(OH)_2D_3$ (10^{-10} M) alone did not increase the amplification product after 24 hours. (*B*) The RT-PCR amplification products are detected by electrophoresis in a 2% agarose gel and visualized by ethidium bromide staining and ultraviolet transillumination. Results are given as mean ± SEM of the VDR/β-actin mRNA ratio of three separate experiments. Statistics are by ANOVA. *$P < 0.05$ versus control.

binding capacity with prolonged incubation with $1,25(OH)_2D_3$ was not due to an increase of occupied receptors, because addition of 10^{-9} M $1,25(OH)_2D_3$ during the last hour of incubation period did not affect N_{max} nor change K_d (data not given).

In contrast, continuous coincubation of chondrocytes with bPTH(1-34) 10^{-10} M and $1,25(OH)_2D_3$ 10^{-12} M up-regulated the VDR to a similar level as each of the two hormones alone after 24 hours (Figs. 2 and 3). However, for prolonged incubation periods, coincubation with both hormones resulted in a persistent up-regulation of VDR up to 48 hours (Fig. 3).

The effect of bPTH(1-34) (10^{-10} M) and $1,25(OH)_2D_3$ (10^{-12} M) or their combination on VDR mRNA is shown in Figure 4. RT-PCR yielded a 297 bp amplification product for the VDR mRNA and a 405 bp product for β-actin mRNA (Fig. 4B). A significant increase of VDR mRNA was observed after 24 hours incubation with bPTH(1-34), regardless whether coincubated with $1,25(OH)_2D_3$ (10^{-12} M) or not. In the group treated with $1,25(OH)_2D_3$ (10^{-12} M), no difference in VDR mRNA levels were found at 24 hours compared to control levels (Fig. 4A).

DISCUSSION

The present study confirms that $1,25(OH)_2D_3$ is stimulatory on DNA synthesis of and colony formation by chondrocytes at low concentrations and is inhibitory at high concentrations [9]. These concentrations of $1,25(OH)_2D_3$ are effective not only in 10% charcoal-stripped FCS but also in serum-free medium (data not shown).

PTH as well as $1,25(OH)_2D_3$ have stimulatory effects on chondrocytes as evidenced by (*i*) increased DNA synthesis (radiothymidine incorporation in monolayer cultures) and (*ii*) increased cloning efficiency (that is, colony formation in agarose stabilized suspension cultures). Maximal effective concentrations were identical to those established previously in dose response studies for cell proliferation in a monolayer culture system [9, 13, 14] using aminoterminal [bPTH(1-34)] and mid-regional PTH fragments [hPTH(28-48)] and $1,25(OH)_2D_3$, respectively. Coincubation with both agonists at their optimal concentration (that is, 10^{-10} M bPTH(1-34) and 10^{-12} M $1,25(OH)_2D_3$, respectively) had additive effects.

The mechanism responsible for delayed homologous down-regulation of VDR is unknown, specifically whether the activity of pre-existing receptor molecules is modulated (that is, phosphorylation), whether VDR interacts with modulating proteins, or whether VDR synthesis is altered on the postranscriptional level. However, the missing up-regulation VDR mRNA concentrations after 24 hours of incubation with $1,25(OH)_2D_3$ indicate, at least in part, a post-transcriptional mechanism. In other systems, that is, fibroblasts and intestinal epithelial cells [23], transfected COS-cells [24], and karatinocytes [25], post-transcriptional regulation of VDR was demonstrated. Down-regulation of VDR may limit the action of vitamin D, since it has been shown that the biological activity of $1,25(OH)_2D_3$ is proportional to VDR expression [26, 27]. Irrespective of the mechanism involved, PTH reversed the effect of $1,25(OH)_2D_3$ on VDR expression, thus rendering possible increased biological activity of $1,25(OH)_2D_3$.

Hyperparathyroidism contributes to the growth disturbance of

uremic children. This is due to the destructive effect of excessive PTH concentrations on metaphyseal cartilage and bone structure that may automatically lead to epiphyseal slipping [1, 28]. This effect contrasts with the action of more physiological concentrations of PTH which are clearly stimulatory. This is shown by the observation of short stature in hypoparathyroid subjects despite adequate vitamin D treatment [29] and is complemented by *in vitro* studies showing a stimulatory effect of PTH on growth cartilage cells [10, 13].

Administration of vitamin D (metabolites) stimulates growth in long-standing nutritional rickets and low-dosed active vitamin D metabolites in calcitriol deficiency, that is, renal failure [3, 30–33]. This contrasts with the antiproliferative effect of high-dose $1,25(OH)_2D_3$ on growth cartilage cells *in vitro* [9] and on abnormal keratinocyte proliferation in psoriasis *in vitro* and *in vivo* [34, 35].

It has been discussed whether high-dose pulse therapy of $1,25(OH)_2D_3$ has a growth depressing effect in uremic children either by substantial lowering of PTH concentrations [36] or by direct antiproliferative effects of $1,25(OH)_2D_3$ on the growth apparatus [9, 37]. The above observation would argue for caution in using $1,25(OH)_2D_3$ pulse therapy for treatment of renal hyperparathyroidism in all uremic children. Studies to define the optimal target PTH concentration are clearly required.

Our finding that PTH is able to maintain up-regulation of the VDR irrespective of continuous exposure of cells to $1,25(OH)_2D_3$ is of more general interest. Previously, we were able to demonstrate that the temporal pattern of exposure of chondrocytes to $1,25(OH)_2D_3$ influences VDR expression, an intermittent mode of exposure maintaining a high VDR level in contrast to continuous incubation with $1,25(OH)_2D_3$ [14]. We speculated that the higher binding capacity for $1,25(OH)_2D_3$ per cell may explain, at least in part, the superior efficacy of intermittent exposure to $1,25(OH)_2D_3$ with respect to suppression of secondary hyperparathyroidism. Several groups determined the VDR status in animals [38] and humans [39] with chronic renal failure. Different results have been reported that are explained, at least in part, by technical difficulties of measuring VDR *ex vivo* [40]. None of the investigators has taken into account so far that not only uremia, the vitamin D status and circulating $1,25(OH)_2D_3$, but also circulating PTH contributes to regulation of the VDR.

In conclusion, PTH has stimulatory effects on growth cartilage chondrocytes that are additive to those of $1,25(OH)_2D_3$, and this may be explained in part by up-regulation of the VDR. The effect of PTH is time-dependent. This raises the issue of whether the spacing of $1,25(OH)_2D_3$ administration is important in uremic children, a point of obvious importance for developing treatment strategies for children with secondary renal hyperparathyroidism.

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